Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1779 (2008) 99–107
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbagrm

Small RNAs in tomato fruit and leaf development

Asuka Itaya [a,b], Ralf Bundschuh [c,d,e], Anthony J. Archual [a,b], Je-Gun Joung [f], Zhangjun Fei [f,g], Xinbin Dai [h], Patrick X. Zhao [h], Yuhong Tang [h], Richard S. Nelson [h], Biao Ding [a,b,d,i,*]

[a] Department of Plant Cellular and Molecular Biology, Ohio State University, Columbus, Ohio 43210, USA
[b] Plant Biotechnology Center, Ohio State University, Columbus, Ohio 43210, USA
[c] Department of Physics, Ohio State University, Columbus, Ohio 43210, USA
[d] The RNA Group, Ohio State University, Columbus, Ohio 43210, USA
[e] Interdisciplinary Biophysics Graduate Program, Ohio State University, Columbus, Ohio 43210, USA
[f] Boyce Thompson Institute, Cornell University, Ithaca, New York 14853, USA
[g] USDA Plant, Soil and Nutrition Laboratory, Ithaca, New York 14853, USA
[h] Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73401, USA
[i] Molecular, Cellular and Developmental Biology Program, Ohio State University, Columbus, Ohio 43210, USA

Received 19 July 2007; received in revised form 31 August 2007; accepted 10 September 2007
Available online 3 December 2007

Abstract

Tomato fruit and leaf development offers excellent systems to study the evolution of gene regulation underlying development of different organs. We have identified over 350 and 700 small RNAs from tomato fruit and leaf, respectively. Except for conserved microRNAs, more than 90% of the small RNAs are unique to tomato. We confirmed expression of some conserved as well as novel putative microRNAs by Northern hybridization. Our results help form a basis for comparative studies on how small RNA-mediated gene expression has contributed to the evolution of common and distinct developmental pathways of fruits and leaves. We have established a website (http://ted.bti.cornell.edu/digital/sRNA/) for public access to all of our small RNA sequences, their expression patterns in respective tissues, and their matching genes or predicted target genes in a searchable manner.

Keywords: Tomato; Small RNA; miRNA; siRNA; Fruit development; Leaf development

1. Introduction

Understanding the mechanisms regulating fruit development has practical and basic importance. Tomato is an excellent model to study fleshy fruit development because of the rich genetic and molecular toolkits available and its economic importance [1]. Its development undergoes several stages [2,3]: i) floral development and fruit set, ii) cell division after anthesis and fertilization, iii) cell expansion, and iv) fruit ripening. The substantial changes in cellular and biochemical events during tomato fruit development allow integrative analyses of many aspects of plant biology. These include hormone biosynthesis and function, cell division control, cell-to-cell communication, cell wall dynamics, and various metabolic pathways [2–5].

Plant hormones [4] and metabolites [5] play important roles in tomato fruit development. Several genes that determine fruit characteristics have been cloned [1]. Transcript expression profiles [6–11] as well as proteome [12,13] are available. Despite these advances, how gene expression is regulated for the development of a fruit remains poorly understood. One aspect of fruit development, which bears great significance in the evolution of plant forms, is the ontogenic relationship between fruits and leaves. Such a relationship is supported by structural similarity, the expression of certain genes in both fruits and leaves, and genetic evidence that some MADS-box genes affect the development of both leaves and fruits [2]. Comparative analyses of gene regulation underlying fruit and leaf development should help further understand the evolution of plant organs.

It is possible that RNA silencing mediated by the 20–24 nt small RNAs such as microRNAs (miRNAs) and small

* Corresponding author. Plant Biotechnology Center, Ohio State, University, 207 Rightmire Hall, 1060 Carmack Road, Columbus, OH 43210, USA. Tel.: +1 614 247 6077; fax: +1 614 292 5379.
E-mail address: ding.35@osu.edu (B. Ding).

1874-9399/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagrm.2007.09.003

interfering RNAs (siRNAs) regulates common and distinct developmental processes in the tomato fruit and leaf, in light of their demonstrated roles in many aspects of plant developmental processes based on studies with model systems such as *Arabidopsis*, maize and rice [14–18]. Small RNAs are produced as duplexes (e.g., miRNA:miRNA* duplex) from precursor RNAs via cleavage by dicer (in animals) or dicer-like (DCL; in plants) RNases. One strand of the duplex is loaded into RISC (RNA-induced silencing complex) to mediate sequence-specific mRNA cleavage or translation inhibition, whereas the other strand (e.g., miRNA*) is degraded [19,20]. Furthermore, small RNAs can mediate chromatin modification to regulate gene expression at the transcriptional level [21,22].

Indeed, miRNAs were identified in tomato by computation [23] and detected in tomato seeds [24,25] and leaves [26] by Northern hybridization, miRNAs and other small RNAs were identified in mature green tomato fruit by sequencing [27], and miR319-regulated expression of *LANCEOLATE* is necessary for compound leaf development in tomato [28]. Here we report our analyses of small RNA profiles in tomato leaf as well as fruit at various developmental stages.

2. Materials and methods

2.1. Cloning and sequencing of tomato small RNAs

The procedure was essentially the same as previously described [29]. Briefly, total RNA was isolated from tomato (*Solanum lycopersicum* cv. Rutgers) leaf and fruit samples. Small RNAs were enriched by polyethylene glycol differential precipitation. Small RNAs of 15–30 nt were gel-isolated from 15% PAGE, ligated to 3′ and 5′ adapters, reverse transcribed and amplified by polymerase chain reaction (PCR). The PCR products were cloned into the TOPO TA vector (Invitrogen, Carlsbad, California) and sequenced at the Plant-Microbe Genomics Facility, Ohio State University and at the Samuel R. Noble Foundation Genomics Facility.

2.2. Small RNA analyses

Adaptor sequences were removed to obtain small RNA sequences and the resulting sequences were filtered for lengths between 15 and 30 nt as described [29]. The small RNA sequences were BLAST searched to identify matching sequences in the following databases: the NBCI Genbank (http://www.ncbi.nlm.nih.gov/Genbank/index.html), the SGN tomato genome and Unigene databases (http://sgn.cornell.edu/), the Arabidopsis small RNA database (http://asrp.cgrb.oregonstate.edu/), the MicroRNA (release 9.2) and MicroRNA hairpin databases (http://microrna.sanger.ac.uk/sequences/), TAIR Arabidopsis genome database (http://www.arabidopsis.org/), TIGR Rice genome database (http://www.tigr.org/tdb/e2k1/osa/), and JGI Popular genome database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). Target gene prediction was performed based on the parameters previously established [30].

2.3. Identification of target gene cleavage

RNA ligase-mediated 5′ RACE (Rapid Amplification of cDNA Ends) was performed on poly-adenylated RNA of respective samples as previously described [31], except that a nested PCR reaction was performed. The sequences of reverse primers used for RT-PCR and nested PCR were as follows. For AP2, the RT primer, TCAAGAAGGTCTCATGAGTGAATG and the nested primer, ATGGAAACCATTTTCTGAGGAC. For AGO1, the RT primer, ACAAGGCCACTGGGTATGCTGAAT and the nested primer, AACAAACCCATAAGTTTCTCG. For ARF8, the RT primer, GGCCCTGTTTGCCCATCTGCTG and the nested primer, GATACTCTCTCCACTTGAAGTG.

2.4. Prediction of precursor genes

The small RNAs were mapped to corresponding regions in SGN BAC, BAC-end and Unigene sequences (http://sgn.cornell.edu/) by BLASTN algorithm. The sequences that matched at least 90% of a given small RNA (with up to one mismatch) were extracted with flanking sequences (110 nt on both sides) and further screened by RNA fold prediction program for their potential hairpin structure(s) [32]. Genes

A

Sequence	Length	Number of reads			
		Leaf	Flower bud	Fruit at 10 DPA	Mature fruit
TGCCTGGCTCCCTGTATGCCA	21	1	0	0	0

B

databases	Number of hits (≤3 mismatch)
miRNA database (release 9.2)	33
miRNA hairpin database	34
Arabidopsis small RNA database	15
GenBank nucleotide database (nt)	150
Tomato finished BAC database (v95)	No hits found
SGN Tomato unigene database	2
Arabidopsis genome - TAIR	4
Rice genome - TIGR	10
Poplar genome - JGI	17

C

mRNA	Alignment	Score	Mismatch	Wobble	Indel	mRNA Direction
SGN-U332548	mRNA (415 – 435) mRNA 5' AGGCAUACAGGGAGCCAGGCA 3' sRNA 3' ACCGUAUGUCCCUCGGUCCGU 5'	1	1	0	0	+
SGN-U324618	mRNA (598 – 618) mRNA 5' GGGCAUGCAGGGAGCCAGGCA 3' sRNA 3' ACCGUAUGUCCCUCGGUCCGU 5'	1.5	1	1	0	+

Fig. 1. Search functions in the tomato small RNA database. (A) and (B) The result page from a query for small RNA SlsmR-301. The page displays the sequences, the abundance in each tissue (A) and the hits from different databases (B) of SlsmR-301. (C) Tomato target genes returned by search using the sequence of SlsmR-301. The score is described in Jones-Rhoades and Bartel [30]. Indel, insertion/deletion.

that contained the small RNA sequences on either stems of hairpin structures were selected as candidate precursor genes.

2.5. Northern blotting of tomato small RNAs

The procedure was essentially the same as previously described [29]. Briefly, 2 to 8 μg of enriched small RNAs (see above) was separated on 17% PAGE/8 M urea/0.5× TBE, transferred to nylon membranes and UV crosslinked. The oligo DNA probes corresponding to the small RNAs were 5′ end-labeled by T4 polynucleotide kinase and [γ-^{32}P] ATP. The membranes were hybridized to the probes overnight at 37 °C and washed twice in 2× SSC/0.1% SDS for 15 min. The washed membranes were exposed to Storage Phosphor Screen (Kodak, Rochester, New York). The Screen was scanned by Molecular Imager FX using Quantity One-4.1.1 software (Bio-Rad, Hercules, California).

3. Results

3.1. Cloning and sequencing of small RNAs in tomato fruit and leaf

We cloned and sequenced RNAs of 15–30 nt from tomato mature leaves as well as fruit at three successive stages of development: flower bud, young fruit at 10 days post anthesis (equivalent to early cell expansion stage), and mature ripe fruit. Two biological replicates for each stage of fruit and five replicates for leaves were analyzed. We obtained a total of 1210 nonredundant sequences, among which 761 are expressed in leaf, 105 in flower bud, 123 in young fruit, and 120 in mature fruit.

The tomato small RNAs were designated as "SlsmR-#," where SlsmR stands for *Solanum lycopersicum sm*all *R*NA and the "#" is the unique number for each small RNA. Small RNAs that differ by length or by 1–3 mismatches are grouped into a family. Members of the same family are assigned the same identification number and then differentiated by letters (e.g., SlsmR-1a and SlsmR-1b).

3.2. Establishment of tomato small RNA database

We have established a website for tomato small RNAs (http://ted.bti.cornell.edu/digital/sRNA/) under Tomato Expression Database [TED; 6]. By entering the SlsmR number of a small RNA into the website, its sequence, abundance in specific organ, and matching genes or predicted target genes from 9 databases (see Materials and methods) can be obtained from the website (Fig. 1). We also set up a searching system in which one can search our database by a user-defined small RNA or mRNA sequence to identify identical/similar small RNAs or potential small RNA-mRNA pairs. All of our small RNA sequences are

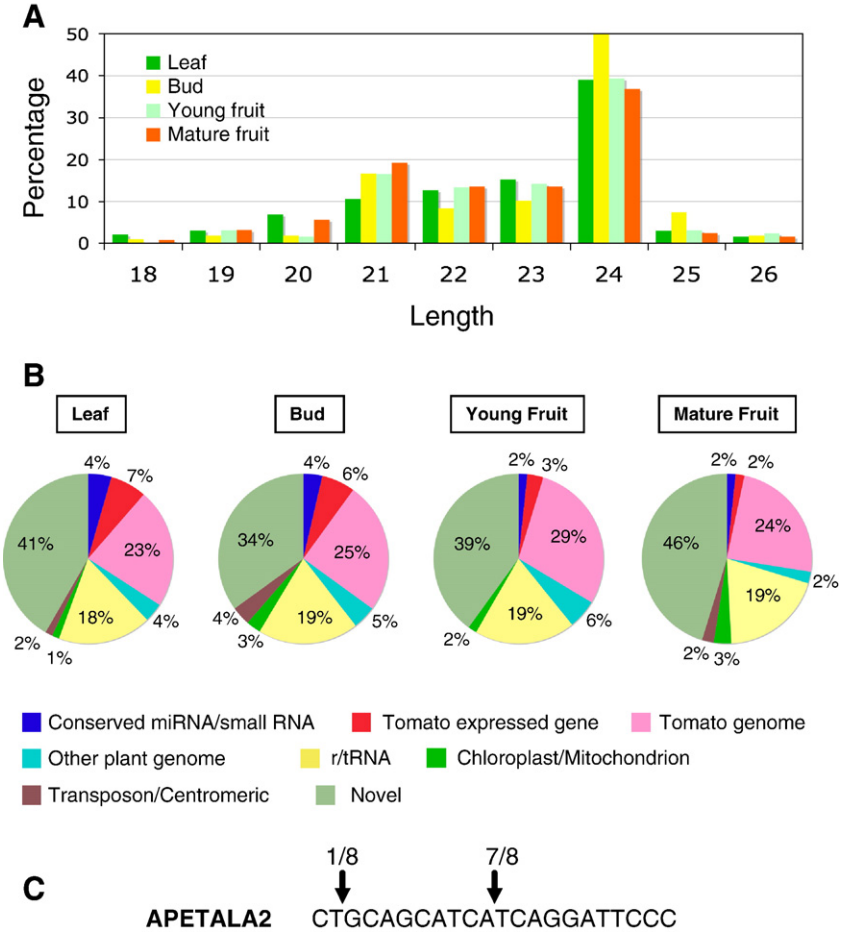

Fig. 2. Characteristics of tomato small RNAs. (A) Size distribution of tomato small RNAs in different organs. (B) Categories of small RNAs in different organs. (C) Site of mir172-mediated cleavage on AP2 mRNA.

also available for downloading from the website. The website is open to the public and new small RNAs can be deposited in the database.

3.3. Size profiles of tomato small RNAs

As shown in Fig. 2A, 80–85% of fruit small RNAs ranged between 21 and 24 nt in length, with 24 nt being the most abundant (37–50%) followed by 21 nt (16–19%). Small RNAs from leaves exhibited similar size profiles, with the exception that the 23 nt small RNAs (15%) were more abundant than the 21 nt (10%).

3.4. Categories of small RNAs

Based on BLAST search results from all current plant databases, we categorized the tomato small RNAs as follows (Fig. 2B): (1) conserved miRNAs/small RNAs (2–4%), (2) expressed tomato genes (2–7%), (3) tomato genomic sequences (23–29%), (4) other plant genomic sequences (2–6%), (5) tRNAs and rRNAs (18–19%), (6) chloroplast and mitochondrion sequences (1–3%), (7) transposons, endogenous paraviruses, or centromeric regions (0–4%), and (8) novel sequences that do not match any current plant DNA sequences (34–46%). Except for conserved miRNAs/small RNAs (see below), 96% of the tomato small RNAs have not been reported from other plant species, suggesting that some tomato small RNAs may play novel regulatory roles.

Surprisingly, if rRNA and tRNA sequences were removed from analyses, only some conserved miRNAs, SlsmR-31a and SlsmR-33b were expressed in more than one organ or stage. All other small RNAs were unique to a particular organ or developmental stage. These patterns, however, remain to be further tested by deep sequencing.

3.5. Conserved miRNAs/small RNAs and their putative target genes

In our small RNA pool, we identified 25 sequences that matched 13 miRNAs and 6 small RNAs reported from other plants (Table 1). We also identified four small RNAs that matched the stem regions of miRNA precursor genes, but not the mature miRNA region (Table 2). Seven tomato miRNAs reported by Pilcher et al. [27], mir159, mir160, mir162, mir164, mir166, mir168 and mir171, were also found in our study. We identified additional miRNAs, mir167, mir169, mir172, mir390, mir424, and mir472. It should be noted that mir424 has not been confirmed experimentally to be a miRNA. In both studies, variants of miRNAs that differ in sequence or by length were found and they may represent family members. Interestingly, these variants

Table 1
Conserved miRNAs and small RNAs

SlsmR-#	Sequence	nt	Number of clones				Description[3]	Predicted target gene	Predicted[4]
			Leaf	Bud	YF[1]	MF[2]	(At smRNA #1)	(SGN-U#)	Precursor
44a	UUUGGAUGAAGGGAGCUCUA	20	1				mir159		
44b	UUUGGAUUGAAGGGAGCUCUA	21	8	2			mir159		
301	UGCCUGGCUCCCUGUAUGCCA	21	1				mir160	ARF 10 (332548)[5]	Yes
1079	UCGAUAAACCUCUGCAUCCAG	21	1				mir162		Yes
48a	UGGAGAAGCAGGGCACAGUGCA	22	1				mir164		
48b	UGGAGAAGCAGGGCACGUGCA	21				1	mir164		
296	UCGGACCAGGCUUCAUUCCCC	21	5				mir166	PHAV (332140)	
298	UGAAGCUGCCAGCAUGAUCUA	21	1		1		mir167*	ARF 8 (340284)	
45a	UCGCUUGGUGCAGGUCGGGAC	21	11			1	mir168	AGO1 (317175)	Yes
45b	UCGCUGGUGCAGGUCGGGA	19				1	mir168	AGO1 (317175)	Yes
292	UAGCCAAGGAUGACUUGCCUG	21	1				mir169*		
124a	UGAUUGAGCCGUGCCAAUC	19	1				mir171	SCR-like 6 (333058)	
124b	UGAUUGAGCCGUGCCAAUAUC	21	1				mir171	SCR-like 6 (333058)	Yes
197a	AGAAUCUUGAUGAUGCUGCAG	21		1			mir172*	AP2 (314858)	
197b	AGAAUCUUGAUGAUGCUGCAU	21	5				mir172*	AP2 (314858)	Yes
197c	GGAAUCUUGAUGAUGCUGCAG	21			1		mir172*	AP2 (314858)	
186	AAGCUCAGGAGGGAUAGCGCC	21	2				mir390*		
271	GGGGAUGUAGCUCAGAUGGUA	21	3	1			mir424*	(330145)	Yes
47a	UCUUUCCUACUCCUCCCAUACC	22	1				mir472*	DRP (337804)	
47b	UCUUUCCUACUCCUCUCAUACC	22	1				mir472*		
114	GACCGCAUAGCGCAGUGGA	19	1				(1680)*		
387	GACAGUUUGGCCGAGUGGUCUA	22	1				(4120)*		
101	AUUCUGGUGUCCUAGGCGU	19	1				(2217 or 14868)*	(330740)	
117	GCGCGGGUAAACGGCGGGA	19	1				(49276)*		
165	GUCUGGGUGGUGUAGUCGGU	20	1				(2800)*		

[1]YF, young fruit at 10 dpa.
[2]MF, mature ripe fruit.
[3]Numbers in parentheses are accession numbers from Arabidopsis small RNA database (http://asrp.cgrb.oregonstate.edu/db/).
[4]See Fig. 3.
[5]ARF, *AUXIN RESPONSE FACTOR*; PHAV, *PHAVOLUTA*; AGO, *ARGONAUTE*; SCR, *SCARECROW*; AP, *APETALA*; DRP, Disease resistance protein.
*miRNA/small RNA that is first cloned and sequenced in tomato in this study.

Table 2
Small RNAs originated from miRNA precursor genes

SlsmR-#	Sequence	nt	Number of clones				Description
			Leaf	Bud	YF[1]	MF[2]	
69	AGGAAAGAGCGAUGGCG	17	1				pre-mir158
64	CAAGUUGAUGGUUGAU	16	1				pre-mir390
122	GUCAGGAUGGCCGAGUGGU	19	2				pre-mir449
114	GACCGCAUAGCGCAGUGGA	19	1				pre-mir457

[1]YF, young fruit at 10 dpa.
[2]MF, mature ripe fruit.

appeared to be expressed in different tissues in our study. This, however, needs to be further examined by deep sequencing.

Martin et al. [25] detected strong hybridization signals for mir167 and mir168 in tomato seeds. We cloned these miRNAs from leaf, young or mature fruit. We did not recover mir319 reported by Ori et al. [28]. Obviously, our cloning has not reached saturation. We could detect, however, mir319 in leaf by Northern hybridization (data not shown).

We identified the putative target genes of three conserved miRNAs in fruit from SGN database (http://sgn.cornell.edu/): AUXIN RESPONSE FACTOR8 (ARF8; SGN340284,mir167 target), ARGONAUTE1 (AGO1; SGN317175, mir168 target) and APETALA2 (AP2; SGN314858, mir172 target). We attempted to verify target gene cleavage by RNA ligase-mediated 5′ RACE [31] and only found AP2 to be cleaved at the same site as reported (Fig. 2C; [33]). The reason why we did not detect AGO1 and ARF8 cleavage is currently unknown. They may not be the bona fide targets, or alternatively, they may be regulated at transcriptional or translational levels in tomato.

3.6. Predicted precursor genes and putative tomato miRNAs

To identify new, putative tomato miRNAs, we searched for small RNA precursor genes in the SGN database. Small RNAs of rRNA or tRNA origin were removed from this analysis. We obtained 11 putative precursor genes, including those for mir160, mir162, mir168, mir171, mir172 and mir424 (Fig. 2). Pilcher et al. [27] also reported precursor genes for mir168 and mir171. Their pre-mir168 differs from ours by a 5 nt-deletion in the stem region and their pre-mir171 is a different gene from ours.

We identified precursor genes for SlsmR-33c, SlsmR-40b and SlsmR-51a. We also noticed that SlsmR-596 (23 nt) contains sequence of the putative-miRNA 2 (18 nt) reported by Pilcher et al. [27]. Therefore, we selected these small RNAs as putative miRNAs. In contrast to Pilcher et al., we were not able to predict reasonable precursor genes for SlsmR-596. Two related small RNAs, SlsmR-31a and 31b, were also selected as putative miRNAs because of their high abundance (especially 31a), which is typical for conserved miRNAs. In addition, SlsmR-31a was one of the only two small RNAs expressed in multiple organs (leaf and young fruit) besides the conserved miRNAs as discussed above. However, we did not identify precursor genes for these small RNAs due to lack of matching sequences in the current databases. All putative miRNAs are listed in Table 3.

3.7. Expression analysis of tomato small RNAs

We could confirm, by Northern hybridization, the expression of all of the conserved miRNAs (except for mir424, data not shown) cloned from flower bud or fruit (Fig. 4A) as well as the putative novel tomato miRNAs (Fig. 4B). Most of the conserved miRNAs and SlsmR-31a showed differential expression among organs (Fig. 4). For example, mir159 is most abundant in bud, mir164 is enriched in mature fruit, and mir167 and SlsmR-31a are greatly reduced in mature fruit. Interestingly, SlsmR-40b and SlsmR-596 displayed two size populations.

Table 3
Putative tomato miRNAs

SlsmR-#	Sequence	nt	Number of clones				Predicted[3]
			Leaf	Bud	YF[1]	MF[2]	Precursor
31a	ACGCAGGAGAGAUGAUGCUGGA	22	17		1		No
31b	CGCAGGAGAGAUGAUGCUGGA	21	2				No
33c	AAGCUAGUGUCACGUAGGCCA	21			1		Yes
40b	AGAUAGUGUCAUGUAGGCCGA	21	1				Yes
51a	CAAGAUAGUGCCACGUAGGUCGA	23	1				Yes
596	UCU**GUAUGUUGGGCCCAAGCC**UA[4]	23	1				?[5]

[1]YF, young fruit at 10 dpa.
[2]MF, mature ripe fruit.
[3]See Fig. 2.
[4]The sequence in bold was reported by Pilcher et al. [23].
[5]Pilcher et al. reported a putative precursor gene for the 19 nt small RNA, but our analysis did not identify a precursor gene for the 23 nt small RNA.

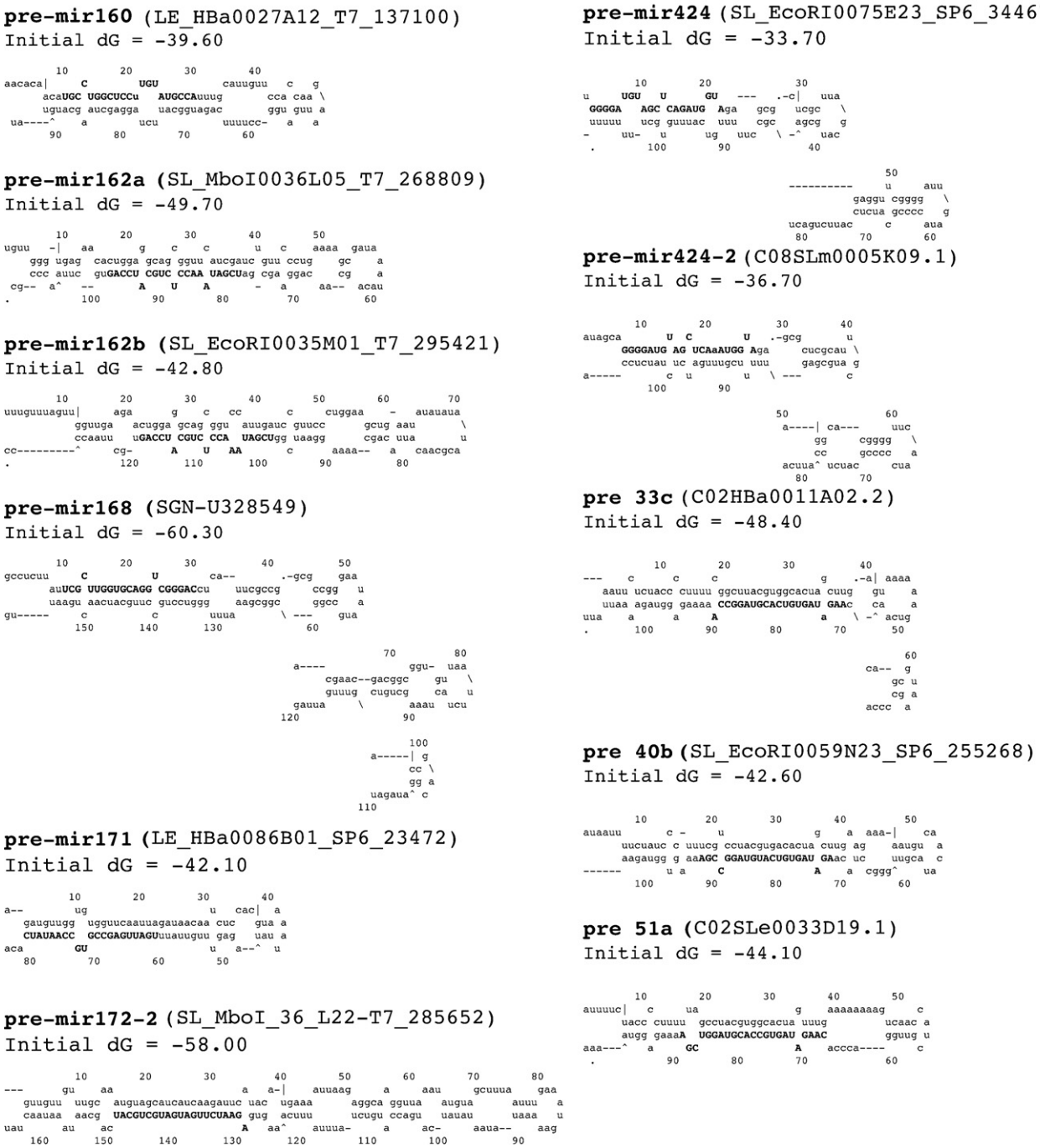

Fig. 3. Secondary structures of predicted precursors for tomato miRNAs or small RNAs, the sequences of which are capitalized.

3.8. Small RNAs of special interest

Twenty-one small RNAs matched chloroplast-encoded genes and six matched mitochondrion-encoded genes. Although many are of rRNA or tRNA origin, some are intergenic and some match chloroplast-encoded psbA gene (Table 4). Five small RNAs (SlsmR-128, -53b, -855, -902, and -989) from different tissues matched polygalacturonase genes that are important for cell wall metabolism. SlsmR-55b and -485 from mature fruit and leaf, respectively, matched Lefsm1 (fruit SANT/MYB-like 1) that was involved in fruit development [34]. Many different small RNAs matched resistance genes or loci (Table 5). The above small RNAs are of either antisense or sense orientation relative to the corresponding genes, and some small RNAs originate from different regions of the same gene.

4. Discussion

Pilcher et al. [27] recently reported small RNAs from mature green fruit of tomato. Our analyses were extended to include flower buds, young and ripe fruit as well as leaves. Therefore, the two studies complement each other and enrich our pool of

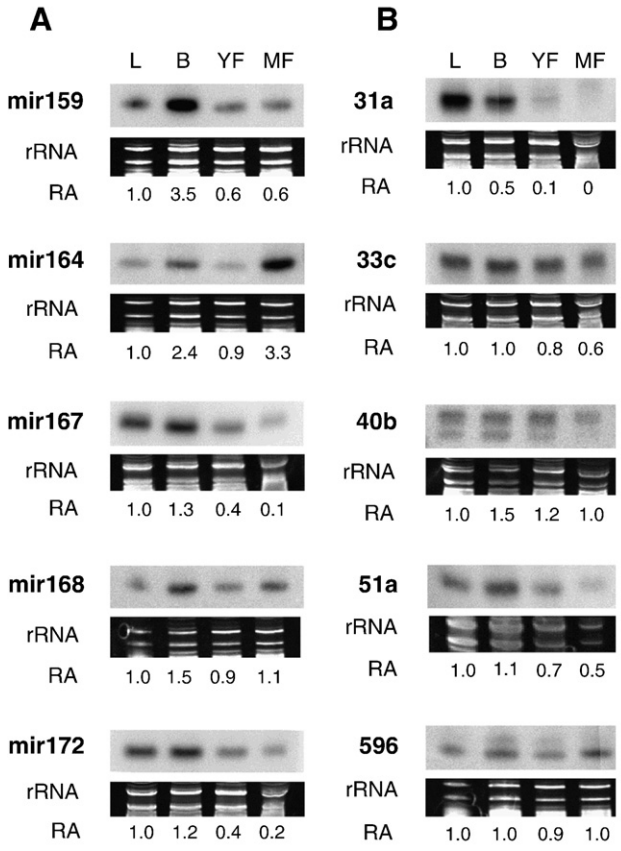

Fig. 4. Northern hybridization of tomato small RNAs in different organs. (A) Conserved miRNAs cloned from flower bud or fruit and (B) putative miRNAs. Accumulation levels of a small RNA in different organs are expressed relative to that in the leaf (arbitrarily set to 1.0). L: leaf, B: flower bud, YF: young fruit, MF: mature ripe fruit, RA: ratio of accumulation levels obtained from two biological replicates.

small RNAs from tomato. We identified common as well as different small RNAs in comparison with Pilcher et al. [27]. One prominent difference is the size profile, with 18–22 nt species being dominant in their study whereas 21–24 nt species are dominant in ours. Notably, the 24 nt species is the most abundant in our collection. The differences may be attributed to the different fruit developmental stages, tissues or even tomato varieties used (i.e., Pilcher et al. used pericarp of mature green fruit in cv. Ailsa Craig whereas we used whole fruit at other stages as well as leaf in cv. Rutgers). Deep sequencing is necessary to establish a concrete profile. As a common problem with all small RNA sequencing data, we cannot exclude the possibility that some small RNAs in our current collection represent degradation products. Nonetheless, all data combined establish a firm foundation for further studies on in-depth sequencing of tomato small RNAs and on experimental testing of their roles in the development of tomato fruit and leaves.

We identified mir160, mir164 and mir172 that are involved in flower identity specification as well as leaf development, mir167 that is involved in auxin response, and mir159 that is involved in flowering time in other plant species [15]. Mir390 is required for *trans*-acting (ta)-siRNA production in *Arabidiopsis* [35]. The conserved miRNAs may be expected to play similar regulatory roles in tomato.

We have identified six putative novel tomato miRNAs whose target genes are unknown. Thus, their biochemical nature and function remain to be determined. All of the putative miRNAs as well as the conserved miRNAs cloned from flower bud or fruit were confirmed to be expressed. The differential accumulation levels of some of these small RNAs in different organs or in the same organ at different stages suggest their distinct regulatory roles. We also tested cleavage of 11 putative target genes of some small RNAs in the fruit but were unable to detect cleavage products (data not shown). More comprehensive studies will be necessary to determine whether these are bona fide target genes regulated by the small RNAs at different levels. For most small RNAs, their precursors and target genes cannot be predicted at this stage because of the limited tomato genome sequence information.

Lefsm1 is a SANT/MYB protein, expressed specifically at the very early stage of fruit development [34]. Presence of small RNAs against Lefsm1 in leaf and mature fruit raises the possibility that RNA silencing is one of the mechanisms to regulate temporal and spatial expression of this gene. Cell wall metabolism is critical for fruit ripening [5]. Fruits become more susceptible to pathogen infection during maturation [3]. It will be of great interest to determine whether small RNAs that match cell wall metabolism and disease resistance genes regulate these processes. The identification of small RNAs matching chloroplast or mitochondrion genome is intriguing. Chloroplast and mitochondrion small RNAs were also reported from *Chlamydomonas reinhardtii* [36]. There is currently no information about small RNA biogenesis in these organelles. Therefore, either there is a novel small RNA biogenesis pathway in these

Table 4
Chloroplast and mitochondrion small RNAs

SlsmR-#	Sequence	nt	Number of clones				Organelle	Annotation	GI #	Polarity
			Leaf	Bud	YF[1]	MF[2]				
166	GUUACGAAGGUGUAGCGGAA	20				1	Chloroplast	Intergenic	113531108	+
206	AGGAUGGAAAAAGGAACUCCA	21				1	Chloroplast	Intergenic	113531108	−
670	ACCGAGCCGGAUCUGGUGUAUCAU	24		1			Chloroplast	Intergenic	113531108	+
209	AGGCGUAGCUGGGGUAUUCGG	21	1				Chloroplast	psbA gene	84371962	−
794	AUGCGACAUUGGAUUGCUGUUGCA	24	1				Chloroplast	psbA gene	82395421	+
222	AUGUGGUGCAGUGGAUGAGAC	21	1				Mitochondrion	Intergenic	56806513	−

[1]YF, young fruit at 10 dpa.
[2]MF, mature ripe fruit.

Table 5
Small RNAs of special interest

SlsmR-#	Sequence	nt	Number of clones				Description	GI # or	Polarity
			Leaf	Bud	YF[1]	MF[2]		SGN-U #[5]	
902	GCCCACAAGAUAGUGCCAUGUAAG	24	1				TAPG1[3]	2459810	−
989	UGAGGUGGGAUGUUAAAGAAUGAC	24				1	TAPG1	2459810	−
855	CUUUCGAACUAGUGCCACGUAGGC	24				1	TAPG2	2459812	−
53b	GUUGGGCCCACAAGAUAGUGGCCA	24				1	TAPG2	2459812	+
128	AACCCACAAGAUAGUGCCAA	20	1				TAPG4	2459814	−
485	AUAAGUGUGUCUCUGAGAUUUCA	23	1				Lefsm1[4]	36783451	−
55b	AUUGGAACCACAAGAUAGUGUCA	23				1	Lefsm1	36783451	−
231	CGAUCACUUGGCCUGACGUCU	21	1				Disease resistance protein	**317189**	+
263	GGACUCUAUCCUACGAUGUGU	21	2				Disease resistance protein	**317189**	+
500	CAUCACAUUUAACUCCAUCCCAC	23	1				Disease resistance protein	**317189**	+
425	UCCCAACACACUUGCAGCCAGA	22	1				Disease resistance protein	**326028**	+
730	AGGGGGGUCCCAACAUCGGGUGAG	24	1				Disease resistance protein	**326200**	+
199	AGAAUUCAGGAUGAGAUUGCA	21	1				Disease resistance protein	**328255**	+
227	CAUCAAGGUAUCUACGACCUA	21		1			Disease resistance protein	**338296**	+
157	GGAGGAGUUGGCCUAACAUU	20	1				Disease resistance protein	**340282**	+
295	UCCAGAAAUUGUCGCCUUGGA	21	1				Disease resistance protein	**340282**	−
191	ACACGUAAGGAGAAUGUUGCC	21	1				Disease resistance protein	**343011**	+
243	CGUUACAAAAUGGCUCGCAUA	21		1			Mildew resistance protein	63033591	+
146	CUGGCAGAGUGCUCUCGUCC	20	1				Nematode resistance-like	37781279	+
987	UCUUGCUGAGGUAAACCGCUACAA	24	1				Nematode resistance-like	37781285	+
47a	UCUUUCCUACUCCUCCCAUACC	22	1				Resistance gene analog	75914634	+
47b	UCUUUCCUACUCCUCUCAUACC	22	1				Resistance gene analog	75914634	+
453	AAGAUAGUGCCACGUAGGCCCCA	23			1		Resistance gene cluster	9587171	+
979	UCAAAUUUCACAAUGAAACAUGGU	24	1				Resistance gene cluster	9587171	−
58a	GGAGUCACGGAGUGUGCCACAUAA	24	2				Resistance gene cluster	2792183	+
58b	GGAGUCAUGGAGUGUGCCACGUAA	24		1			Resistance gene cluster	2792183	+
879	GAGAUGAGGUGUCUAGAUGUGCAG	24			1		Resistance gene locus	2826843	+
131	ACAUGGGAGGUUCACACAAG	20				1	Resistance protein	15418711	+
214	AGUUCUCUGCCAAGUGUCUCU	21		1			Resistance-like gene	15425949	−
176	UUGACCUACGUAGCACUACA	20	1				Resistance-like protein	115381095	+
464	ACAAGAUAGUGCCACGUAGGCCG	23	1				ToMV-resistance locus	33330975	−
904	GCGCAAGAUAGUGCCACGUAGGCC	24	1				ToMV-resistance locus	33330975	−
666	ACAUACCUAGUGUAAUCCCACAGG	24		1			Tospovirus resistance protein	15418711	+
59c	GGGCCACAAGAUAGUGCCACGUAA	24	1				Tospovirus resistance protein	15418711	−

[1]YF, young fruit at 10 dpa.
[2]MF, mature ripe fruit.
[3]TAPG, polygalacturonase.
[4]Lefsm1, Lycopersicon esculentum fruit SANT/MYB-like 1.
[5]Data in bold are SGN-U#.

organelles that we have not discovered or the small RNAs generated in the nucleus/cytoplasm are imported into these organelles via a mechanism that remains to be identified. Furthermore, whether such small RNAs have any functional significance is yet to be tested.

The presence of some miRNAs in both leaf and fruit of tomato lends further support to the ontogenic relationship between these organs postulated based on similarities in anatomical features and some gene expression patterns [2]. More importantly, our results should allow experimental studies to address the specific roles of small RNAs in regulating common and distinct processes of fruit and leaf development in tomato and other Solanaceous species.

Acknowledgments

We thank Esther van der Knaap and Yijun Qi for the discussions. This work was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (2004-35304-15005 to BD) and by the National Science Foundation (IBN-050515745 and IOB-0620143 to BD and DBI-0501778 to ZF).

References

[1] J.J. Giovannoni, Fruit ripening mutants yield insights into ripening control, Curr. Opin. Plant Biol. 10 (2007) 283–289.
[2] G. Gillaspy, H. Ben-David, W. Gruissem, Fruits: a developmental perspective, Plant Cell 5 (1993) 1439–1451.
[3] J.J. Giovannoni, Genetic regulation of fruit development and ripening, Plant Cell 16 (2004) S170–S180 (Suppl).
[4] A. Srivastava, A.K. Handa, Hormonal regulation of tomato fruit development: a molecular perspective, J. Plant Growth Regul. 24 (2005) 67–82.
[5] F. Carrari, A.R. Fernie, Metabolic regulation underlying tomato fruit development, J. Exp. Bot. 57 (2006) 1883–1897.
[6] Z. Fei, X. Tang, R. Alba, J. Giovannoni, Tomato Expression Database (TED): a suite of data presentation and analysis tools, Nucl. Acids Res. 34 (2006) D766–D770.

[7] Z. Fei, X. Tang, R.M. Alba, J.A. White, C.M. Ronning, G.B. Martin, S.D. Tanksley, J.J. Giovannoni, Comprehensive EST analysis of tomato and comparative genomics of fruit ripening, Plant J. 40 (2004) 47–59.
[8] R. Alba, Z. Fei, P. Payton, Y. Liu, S.L. Moore, P. Debbie, J. Cohn, M. D'Ascenzo, J.S. Gordon, J.K. Rose, G. Martin, S.D. Tanksley, M. Bouzayen, M.M. Jahn, J. Giovannoni, ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development, Plant J. 39 (2004) 697–714.
[9] R. Alba, P. Payton, Z. Fei, R. McQuinn, P. Debbie, G.B. Martin, S.D. Tanksley, J.J. Giovannoni, Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development, Plant Cell 17 (2005) 2954–2965.
[10] S. Moore, P. Payton, M. Wright, S. Tanksley, J. Giovannoni, Utilization of tomato microarrays for comparative gene expression analysis in the *Solanaceae*, J. Exp. Bot. 56 (2005) 2885–2895.
[11] S. Moore, J. Vrebalov, P. Payton, J. Giovannoni, Use of genomics tools to isolate key ripening genes and analyse fruit maturation in tomato, J. Exp. Bot. 53 (2002) 2023–2030.
[12] M. Faurobert, C. Mihr, N. Bertin, T. Pawlowski, L. Negroni, N. Sommerer, M. Causse, Major proteome variations associated with cherry tomato pericarp development and ripening, Plant Physiol. 143 (2007) 1327–1346.
[13] M. Rocco, C. D'Ambrosio, S. Arena, M. Faurobert, A. Scaloni, M. Marra, Proteomic analysis of tomato fruits from two ecotypes during ripening, Proteomics 6 (2006) 3781–3791.
[14] X. Chen, MicroRNA biogenesis and function in plants, FEBS Lett. 579 (2005) 5923–5931.
[15] M.W. Jones-Rhoades, D.P. Bartel, B. Bartel, MicroRNAs and their regulatory roles in plants, Annu. Rev. Plant Biol. 57 (2006) 19–53.
[16] R. Sunkar, J.K. Zhu, MicroRNAs and short-interfering RNAs in plants, J. Integr. Plant Biol. 49 (2007) 817–826.
[17] B. Zhang, X. Pan, T.A. Anderson, Identification of 188 conserved maize microRNAs and their targets, FEBS Lett. 580 (2006) 3753–3762.
[18] B. Zhang, X. Pan, G.P. Cobb, T.A. Anderson, Plant microRNA: a small regulatory molecule with big impact, Dev. Biol. 289 (2006) 3–16.
[19] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[20] H. Vaucheret, Post-transcriptional small RNA pathways in plants: mechanisms and regulations, Genes Dev. 20 (2006) 759–771.
[21] S.W. Chan, I.R. Henderson, S.E. Jacobsen, Gardening the genome: DNA methylation in *Arabidopsis thaliana*, Nat. Rev., Genet. 6 (2004) 351–360.
[22] M.A. Matzke, J.A. Birchler, RNAi-mediated pathways in the nucleus, Nat. Rev., Genet. 6 (2005) 24–35.
[23] B. Zhang, X. Pan, C.H. Cannon, G.P. Cobb, T.A. Anderson, Conservation and divergence of plant microRNA genes, Plant J. 46 (2006) 243–259.
[24] R.C. Martin, P.-P. Liu, H. Nonogaki, Simple purification of small RNAs from seeds and efficient detection of multiple microRNAs expressed in *Arabidopsis thaliana* and tomato (*Lycopersicon esculentum*) seeds, Seed Sci. Res. 15 (2005) 319–328.
[25] R.C. Martin, P.-P. Liu, H. Nonogaki, MicroRNAs in seeds: modified detection techniques and potential applications, Can. J. Bot. 84 (2006) 189–198.
[26] M.A. Denti, A. Boutla, M. Tsagris, M. Tabler, Short interfering RNAs specific for potato spindle tuber viroid are found in the cytoplasm but not in the nucleus, Plant J. 37 (2004) 762–769.
[27] R.L.R. Pilcher, S. Moxon, N. Pakseresht, V. Moulton, K. Manning, G. Seymour, T. Dalmay, Identification of novel small RNAs in tomato (*Solanum lycopersicum*), Planta 226 (2007) 709–717.
[28] N. Ori, A.R. Cohen, A. Etzioni, A. Brand, O. Yanai, S. Shleizer, N. Menda, Z. Amsellem, I. Efroni, I. Pekker, J.P. Alvarez, E. Blum, D. Zamir, Y. Eshed, Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato, Nat. Genet. 39 (2007) 787–791.
[29] A. Itaya, X. Zhong, R. Bundschuh, Y. Qi, Y. Wang, R. Takeda, A.R. Harris, C. Molina, R.S. Nelson, B. Ding, A structured viroid RNA serves as a substrate for dicer-like cleavage to produce biologically active small RNAs but is resistant to RNA-induced silencing complex-mediated degradation, J. Virol. 81 (2007) 2980–2994.
[30] M.W. Jones-Rhoades, D.P. Bartel, Computational identification of plant microRNAs and their targets, including a stress-induced miRNA, Mol Cell 14 (2004) 787–799.
[31] C. Llave, Z. Xie, K.D. Kasschau, J.C. Carrington, Cleavage of scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA, Science 297 (2002) 2053–2056.
[32] I.L. Hofacker, W. Fontana, P.F. Stadler, L.S. Bonhoeffer, M. Tacker, P. Schuster, Fast folding and comparison of RNA secondary structures, Monatsh. Chem. 125 (1994) 167–188.
[33] K.D. Kasschau, Z. Xie, E. Allen, C. Llave, E.J. Chapman, K.A. Krizan, J.C. Carrington, P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA unction, Dev. Cell 4 (2003) 205–217.
[34] R. Barg, I. Sobolev, T. Eilon, A.G.I. Chmelnitsky, S. Shabtai, E. Grotewold, Y. Salts, The tomato early fruit specific gene *Lefsm1* defines a novel class of plant-specific SANT/MYB domain proteins, Planta 221 (2005) 197–211.
[35] E. Allen, Z. Xie, A.M. Gustafson, J.C. Carrington, MicroRNA-directed phasing during trans-acting siRNA biogenesis in plants, Cell 121 (2005) 207–221.
[36] T. Zhao, G. Li, S. Mi, S. Li, G.J. Hannon, X.J. Wang, Y. Qi, A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*, Genes Dev. 21 (2007) 1190–1203.